Effect of Healing on the Expression of Transforming Growth Factor βs and their Receptors in Chronic Venous Leg Ulcers

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The transforming growth factor βs are of major importance in the wound repair process; however, no studies to date have investigated the role of the transforming growth factor β receptors in chronic venous leg ulcers or what effect healing has on these proteins. To determine whether the transforming growth factor β peptides and their receptors are expressed in chronic venous wounds, we used immunofluorescent analysis and quantitative competitive reverse transcription polymerase chain reaction to identify the protein and mRNA expression, respectively. Biopsy samples from wounds and normal skin were collected from 12 patients with chronic venous leg ulcers and three patients undergoing reconstructive surgery, respectively. Additionally four of the chronic venous leg ulcer patients were re-biopsied between 2 and 8 wk after the first biopsy when the wounds had entered the healing phase. The tissue excised from the ulcers included the surrounding intact skin, the ulcer edge, and the ulcer base. Immunofluorescent staining for transforming growth factors β1, β2, and β3 was observed within the epidermis of the skin surrounding the chronic venous ulcers and in fibroblasts and inflammatory cells of the dermis, although this staining was not as strong as that seen in normal unwounded skin. Very little staining could be seen within the ulcers for any of the ligands, however. In contrast the transforming growth factor β type I receptor was observed throughout the ulcers and the normal unwounded skin biopsies, particularly in the basal epidermal cells. No immunofluorescence for the type II transforming growth factor β receptor was observed in any of the ulcer biopsies investigated, although it was observed throughout the epidermis and in fibroblasts and inflammatory cells in the surrounding skin. Quantitative, competitive reverse transcription polymerase chain reaction was used to analyze mRNA expression for transforming growth factor β1 and the type II receptor in the nonhealing ulcers and normal unwounded skin biopsies. These studies revealed that transforming growth factor β1 and transforming growth factor β receptor II mRNA was expressed in all the chronic nonhealing ulcers albeit at very low levels for the type II receptor. In marked contrast to the staining observed in nonhealing chronic ulcers, positive immunostaining was observed for the transforming growth factor βs and both the type I and type II receptors in healing ulcers. These results suggest that the absence of a viable receptor complex for the transforming growth factor βs in nonhealing chronic venous ulcers may contribute to wound chronicity. ***Key words: healing/immunofluorescence/RT-PCR/wound. J Invest Dermatol 117:1282–1289, 2001***

Venous ulceration is the result of chronic venous insufficiency leading to increased pressure in the venous system (Angle and Bergan, 1997). The link between venous hypertension and venous ulceration is incompletely understood, however. It has been suggested that extravasation of plasma proteins including fibrinogen, which polymerizes around dermal capillaries to form "fibrin cuffs", impedes the diffusion of oxygen and nutrients to the dermis and inhibits capillary sprouting. These cuffs have been shown to contain other serum proteins including α2-macroglobulin and factor XIIIa as well as the matrix proteins laminin, fibronectin, tenascin, and collagens I and III, suggesting that components of the cuffs are actively synthesized by surrounding connective tissue cells (Herrick *et al*, 1992). Alternatively, increased pressure in the venous system, which reduces the perfusion pressure and capillary flow rate, may result in the trapping of white blood cells in distal leg capillaries (Coleridge Smith *et al*, 1988). It is proposed that these trapped leukocytes become activated and release toxic metabolites, enzymes, and cytokines, damaging endothelial cells and allowing passage of plasma proteins. In addition, the trapped leukocytes may cause local areas of ischaemia.

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Abbreviations: TGF, transforming growth factor.

The role of growth factors in chronic venous wound healing is poorly understood. Several studies have demonstrated decreased growth factor activity in the chronic wound environment that may contribute to reduced amounts of extracellular matrix synthesis and collagen production (Cooper *et al*, 1994; Higley *et al*, 1995). The colocalization of serum binding proteins and growth factors, e.g., α2-macroglobulin and transforming growth factor β (TGF-β), in fibrin cuffs has led to the suggestion that the trapping or inactivation of growth factors essential to the wound repair process renders them unavailable to the cells of wound repair (Falanga, 1993; Higley *et al*, 1995). Other studies suggest that the pro-inflammatory environment of the ulcer promotes the degradation of growth factors and their receptors (Wallace and Stacey, 1998). Agren *et al* (1999) have revealed that venous-ulcer-derived fibroblasts in culture have reduced growth activity and are less responsive to platelet-derived growth factor. Additionally, *in vitro* studies have revealed venous-leg-ulcer-derived fibroblasts are unresponsive to TGF-β1 and this is associated with reduced levels of the type II TGF-β receptor (Hasan *et al*, 1997).

In normal acute wounds, studies have revealed the importance of the TGF-βs to stimulate the synthesis of multiple extracellular matrix components, including collagens, fibronectin, vitronectin, tenascin, and proteoglycans, reviewed by O'Kane and Ferguson (1997). Additionally they suppress matrix degradation by down-regulating the expression of proteases, such as plasminogen activators (Laiho *et al*, 1986), collagenase (Edwards *et al*, 1987), and stromelysin (Kerr *et al*, 1990), and induce protease inhibitors, such as plasminogen activator inhibitor 1 (Laiho *et al*, 1986; 1987) and tissue inhibitor of metalloproteases (Edwards *et al*, 1987). Numerous studies have shown that exogenously applied TGF-βs enhance and accelerate acute wound healing (Mustoe *et al*, 1987; Shah *et al*, 1995), especially in situations where the normal healing process is naturally or artificially impaired (Cox *et al*, 1992). TGF-β1 and TGF-β3 are both expressed in the thickened epidermis of both acute wounds and decubitus ulcers indicating a possible role in epidermal maintenance and reepithelialization (Schmid *et al*, 1993).

The action of the TGF-βs is mediated by a complex formed through binding to two specific high-affinity transmembrane serine–threonine kinases (Ebner *et al*, 1993). The type I receptor requires the type II receptor to bind ligand, whereas the type II receptor requires the type I receptor for signaling (Wrana *et al*, 1992; Inagaki *et al*, 1993). Expressions of both the type I and type II TGF-β receptors are increased in response to acute wounding (Frank *et al*, 1996), and it has been suggested that excessive scarring may be associated with failure to eliminate TGF-β receptor over-expressing fibroblasts during granulation tissue remodeling, leading to overproduction of matrix proteins and subsequent fibrosis (Schmid *et al*, 1998).

Despite significant investment in the clinical trialing of the TGF-β isoforms on chronic wounds, to date no studies have investigated TGF-β receptor expression in chronic venous leg ulcers. Additionally, no studies have determined the effect of healing on the growth factor and receptor expression in these wounds. The aim of this study was to describe the expression of the TGF-β isoforms and their receptors in the wound bed and margin of the chronic venous ulcer and compare this expression to that seen in normal skin. Additionally the effect of healing on the growth factor and receptor profile was also assessed.

MATERIALS AND METHODS

Venous leg ulcer biopsies and normal skin samples Skin samples were collected from a total of 12 patients with chronic venous leg ulcers on their first presentation at the wound healing clinic at the Queen Elizabeth Hospital, Adelaide. Informed consent was obtained from individual subjects for all procedures that were approved by the Ethics of Human Research Committee of the Queen Elizabeth Hospital, Adelaide. The diagnosis of venous ulcer was based on an ankle brachial index of ≥ 0.7, a toe pressure of ≥ 40 mmHg on the limb of the target ulcer, and absence of diabetes. Patients were between 47 and 86 y of age and ulcer duration ranged from 18 mo to 9 y. Biopsies (10×3 mm) were taken under local anesthetic from the margins of the chronic venous ulcers and included wound margin epithelium and surrounding dermal and epidermal tissue. Wound margin biopsies were embedded in (Tissue-Tek) OCT compound (Sakura Finetek, CA) and frozen in liquid nitrogen. In addition, 6 mm punch biopsies were taken under local anesthetic from the center of the ulcers and immediately frozen in liquid nitrogen for later RNA extraction. All ulcer samples were stored at –70°C until required. Four of the patients consented to an additional biopsy being taken within 2–8 wk after the initial presentation when the ulcers had entered the healing phase as determined by measuring the area of the wound, the appearance of healthy granulation tissue, and increased epithelial coverage of the wound. The ulcers had reduced in size to between 87% and 18% of their original wounded area. During weekly visits to the clinic the wounds were washed using a Medisponge (LMI, MI) containing 0.25% chlorohexidene; moisturizer was applied to the intact skin, blunt debridement was performed as necessary, and the wounds were dressed with Profore (Smith and Nephew, U.K.) according to the manufacturer's directions. Normal skin samples ($2 \text{ cm} \times 1 \text{ cm}$) were obtained from three patients undergoing reconstructive plastic surgery. Immediately upon removal from the patients, half of the tissue was frozen in liquid nitrogen, and the other half was embedded in OCT compound and frozen as before.

Immunofluorescence studies Indirect immunofluorescent localization was used with a biotin–streptavidin amplification step for increased sensitivity. Serial 7 μm cryosections were washed with phosphate-buffered saline (PBS) and incubated for 1 h at room temperature with TGF-β1, TGF-βRI, TGF-βRII primary antibodies (all Santa Cruz Biotechnology, CA; 1:100) or TGF-β2, TGF-β3 (R&D Systems, MN; 1:100) for 1 h at room temperature. After three washes in PBS, sections were incubated with either biotinylated sheep antirabbit (Sigma-Aldrich, Sydney, Australia; 1:200) or biotinylated rabbit antigoat (Zymed Laboratories, CA; 1:200) for 1 h at room temperature. After further washing with PBS, CY3-conjugated streptavidin (Sigma-Aldrich; 1:500) was applied to all sections for 1 h. After final washes in PBS, slides were mounted in Immunmount (Shandon, PA) and examined using a fluorescent microscope. CY3 emits fluorescence at 570 nm, which is visualized as red staining in positive regions. Images were captured and viewed using Image-Pro Plus V3.1 software (Media Cybernetics, MD). For verification of staining, negative controls included preadsorption of the TGF-β1, TGF-β2, TGF-β3 antibodies with excess amounts of human TGF-β1, TGF-β2, and TGF-β3 (10 ng per ml) or control blocking peptides for TGF-βRI and TGF-βRII (sc-399P, sc-400P; Santa Cruz Biotechnology), for 1 h at room temperature. On additional control sections, primary antibodies were replaced by either normal rabbit IgG or normal goat IgG as appropriate or the primary and secondary antibodies were omitted to determine nonspecific binding. No positive staining for any of the peptides investigated was seen on any of the control sections.

Quantitative competitive reverse transcription polymerase chain reaction (RT-PCR) *Escherichia coli* were transformed with the pEGFTGF plasmid (Tarnuzzer *et al*, 1996), and the plasmid DNA was isolated and linearized for RNA transcription. The RNA was purified by poly-A selection and a template dilution series prepared as described previously (Tarnuzzer *et al*, 1996). A 10-fold dilution series in the range 2×10^9–2×10^4 copies of RNA template per μl was then constructed by diluting the competitive RNA template sample in DEPC water. Primer sequences were synthesized as follows: β-actin, antisense 5′-CTCAATGTCACGCACGATTTC-3′, sense 5′-GTGGGGCGCCCC-AGGCACCA-3′, 540 bp; TGF-β1, antisense 5′-GATGCTGGGCCC-TCTCCAGC-3′, sense 5′-CAAGCAGAGTACACACAGCA-3′, 442 bp; TGF-βRII, antisense 5′-GATCTTGACTGCCACTGTCTC-3′, sense 5′-TGTGTTCCTGTAGCTCTGATG-3′, 430 bp.

Competitive RT-PCR Total RNA was extracted from six ulcer biopsies using a Quickprep Total RNA Extraction kit (Amersham Pharmacia Biotech, Uppsala, Sweden). One microgram was reverse transcribed in a series of 20 μl reactions with known dilutions of pEGFTGF competitive RNA (2×10^4–2×10^9 copies), 250 ng oligo$(dT)_{16}$, and 20 U RNasin. The reaction was heated to 65°C for 15 min in a GeneAmp® PCR System 9700 thermal cycler (PE Applied Biosystems, Norwalk, CT). Following denaturation of the RNA, reverse transcription buffer (50 mM Tris-HCl, pH 8.3, at 25°C, 50 mM KCl, 10 mM dithiothreitol, 0.5 mM spermidine), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 15 U AMV reverse transcriptase, and nuclease-free water to a final volume of 20 μl was added to the reaction. Reverse transcription was performed at 42°C for 60 min followed by 95°C for 10 min. To determine mRNA levels of TGF-β1, TGF-βRII, and β-actin, cDNA products were subjected to

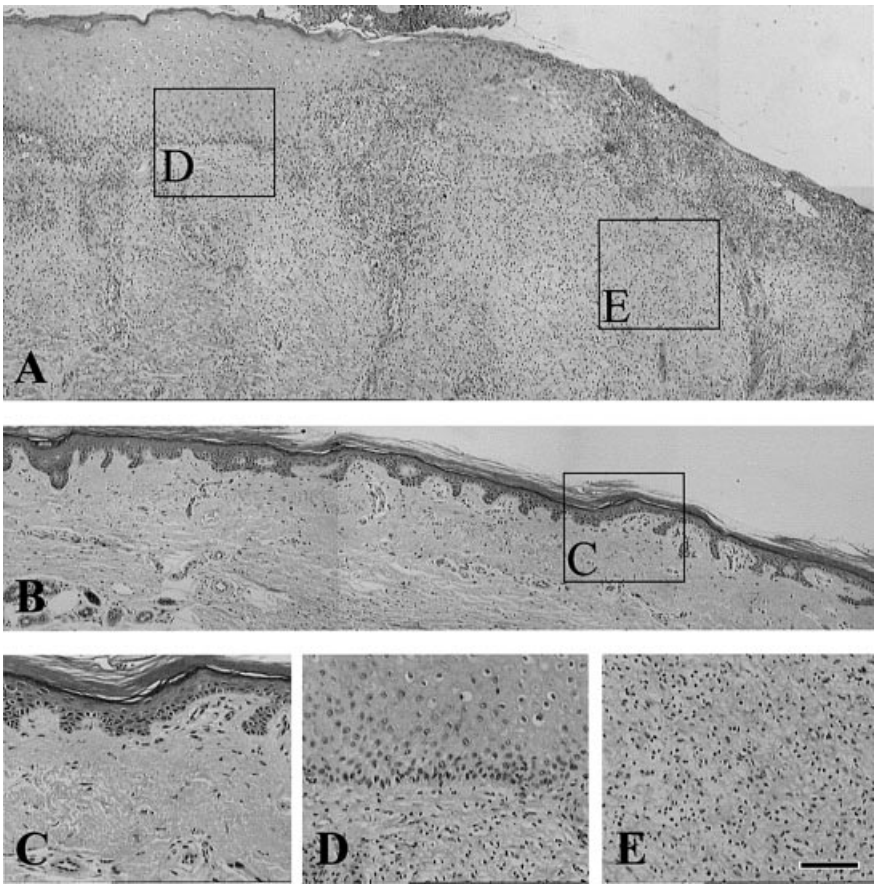

Figure 1. Histology of chronic venous leg ulcers and normal skin. Biopsies from chronic venous leg ulcers and normal skin were fixed in formalin, embedded in paraffin wax, sectioned, and stained with H&E to reveal the cellular morphology of the tissues. Due to the size of the ulcer biopsies a composite picture was made from 15 individual views and a montage created using ImageMaster software (see *Materials and Methods*). A representative montage of a typical venous ulcer is shown in (*A*) with the ulcer margin on the left of the picture. Normal skin is shown in (*B*), and higher magnification pictures are shown of the normal skin (*C*), skin adjacent to the ulcer (*D*), and ulcer tissue (*E*). The boxes in (*A*) and (*B*) indicate the position of the higher magnification pictures. *Scale bar*: (*A*, *B*) 200 μm, (*C–E*) 50 μm.

PCR using primers described earlier. DNA amplification was performed in a 50 μl volume containing 2 μl of the reverse transcription reaction, 1 × PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 9.0, 0.1% Triton®X-100), 1.5 mM $MgCl_2$, 200 μM dNTPs, 50 pmol of each antisense and sense PCR primer, and 2.5 U Taq polymerase. PCR conditions were 94°C for 5 min, followed by 41 cycles of 94°C for 1.5 min, 58°C for 2 min, 72°C for 3 min, and 72°C final extension for 10 min. PCR products were separated on 1.5% agarose gels containing 25 ng per ml ethidium bromide at 90 V for approximately 1.5 h. The gels were visualized under ultraviolet light, and images were captured using an ImageMaster® VDS thermal imaging system (Amersham Pharmacia Biotech). The integrated optical density of bands was determined using ImageMaster VDS software (Amersham Pharmacia Biotech). Intensity values were normalized for their molecular weight, and the log of the ratio of the band intensities within each lane was plotted against the log of copy number of template added per reaction. The number of molecules of target message was determined where the ratio of template and target band intensities was equal to 1.

RESULTS

All the biopsy sites within the venous ulcers healed without complication. Indeed, biopsy wounds extending through the wound margin were noted to heal more quickly than the ulcer margin itself. An example of a hematoxylin and eosin (H&E) stained ulcer wound margin biopsy is shown in **Fig 1**(***A***). Features characteristic of chronic venous ulcers were noted in all wounds studied. The ulcer wound base was characterized by areas of vascular granulation tissue overlying disorganized collagen bundles. A prominent inflammatory infiltrate was present in most wounds, often extending into the surrounding dermis. Characteristic hemosiderin deposits and capillary cuffing were noted. The epithelial margin was characterized by a marked hyperplastic acanthosis. An example of normal skin stained with H&E is shown in **Fig 1**(***B***). Representative H&E stained views of normal skin, ulcer margin, and ulcer base (**Fig 1*C–E***, respectively) represent the views shown in the subsequent immunofluorescent studies (**Figs 2, 3**). Twelve chronic nonhealing ulcers, four healing ulcers, and three normal skins were examined for TGF-β1, TGF-β2, TGF-β3, TGF-βRI, and TGF-RII, and representative pictures of the staining observed in all of these tissues are shown in **Figs 2** and **3**.

Expression of TGF-β and its receptors in chronic wounds and normal unwounded skin Immunofluorescent stained sections of normal unwounded skin revealed the presence of TGF-β1 particularly throughout the epidermis and within fibroblasts within the dermis (**Fig 2*A***). At the margins of the ulcers, there was obvious TGF-β1 staining in the thickened epidermis, the epithelium of hair follicles, and fibroblasts (**Fig 2*B***). Staining of TGF-β1 within the ulcers was markedly reduced, however (**Fig 2*C***). In addition, TGF-β1 expression in the epidermal margin of the ulcers was reduced compared to that seen in normal epidermis (**Fig 2*A***). In the healing ulcers, positive staining for TGF-β1 was observed particularly around blood vessels and fibroblasts (**Fig 2*D***).

TGF-β2 immunofluorescence showed a different distribution to that observed for TGF-β1. In normal unwounded skin TGF-β2 immunoreactivity was observed in suprabasal epidermal cells and in fibroblasts within the dermis (**Fig 2*E***). In contrast, no staining was observed in the epidermal margin of the ulcers, although fibroblasts and inflammatory cells within the dermal wound margins were TGF-β2 positive (**Fig 2*F***). The intensity of the TGF-β2 staining was markedly reduced within the ulcers compared to normal dermis or the wound margin (**Fig 2*G***). In contrast to the nonhealing ulcers, the healing ulcers showed marked cellular staining for TGF-β2, particularly in fibroblasts within the granulation tissue of the wounds (**Fig 2*H***).

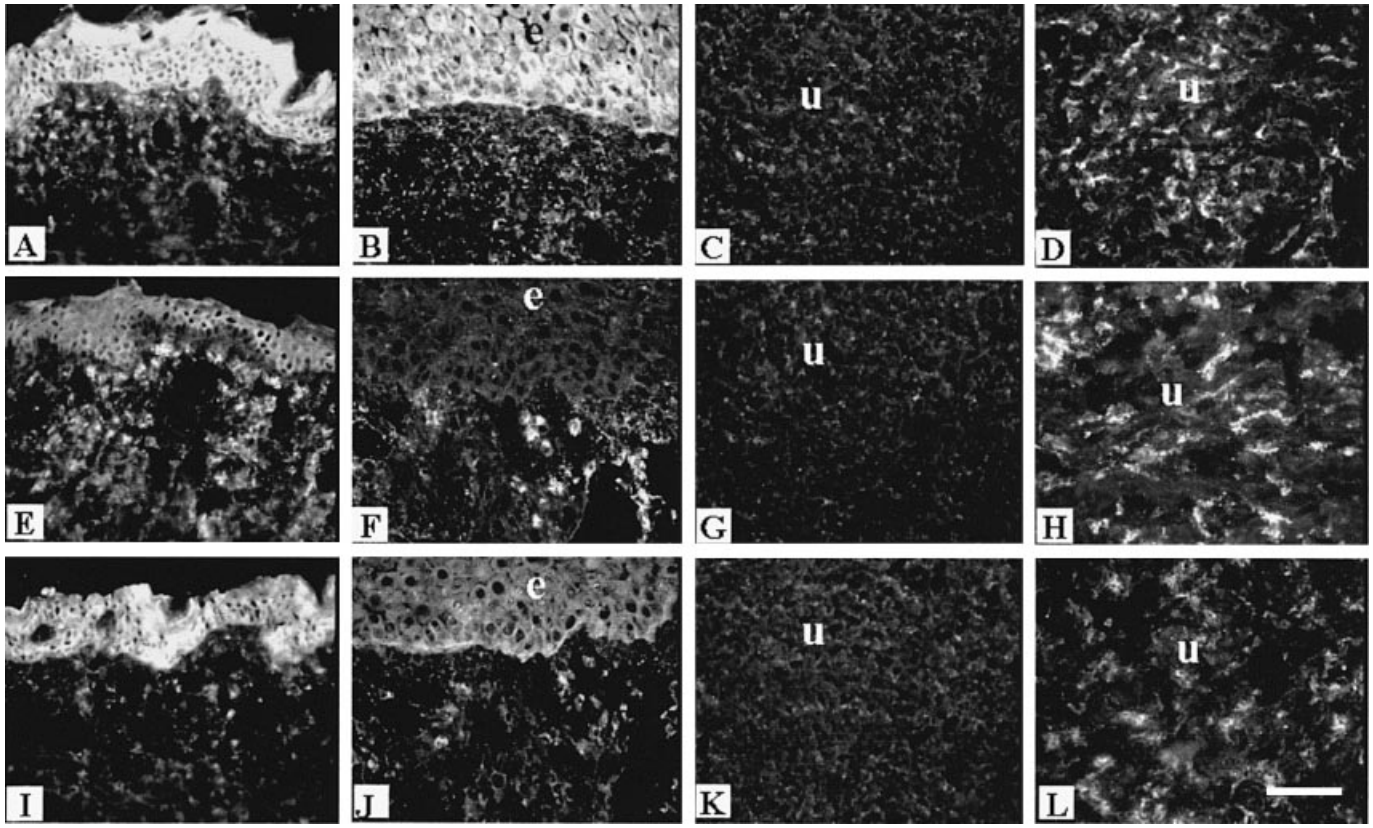

Figure 2. Immunofluorescent staining of the TGF-βs in unwounded normal skin and nonhealing and healing chronic venous ulcers. The unwounded normal skins and ulcer biopsies were stained using antibodies to TGF-β1, TGF-β2, and TGF-β3. Representative staining of normal skin (*A*, *E*, *I*), ulcer edge (*B*, *F*, *J*), nonhealing ulcer (*C*, *G*, *K*), and healing ulcer (*D*, *H*, *L*) is shown. TGF-β1 immunofluorescent staining is shown in (*A*), (*B*), (*C*), (*D*), TGF-β2 in (*E*), (*F*), (*G*), (*H*), and TGF-β3 in (*I*), (*J*), (*K*), (*L*). *Scale bar*: 50 μm.

TGF-β3 immunostaining was observed throughout the epidermis and in fibroblasts in normal unwounded skin (**Fig 2*I***). Reduced epidermal staining was observed at the margins of the ulcers with inflammatory cells staining positive at the dermal ulcer edge (**Fig 2*J***). No staining was observed within any of the nonhealing ulcers investigated (**Fig 2*K***). In contrast, clear positive staining was observed in all of the healing ulcers (**Fig 2*L***).

The expression of TGF-β receptors was examined in biopsies from chronic venous ulcers and compared to that in normal unwounded skin and healing ulcers. TGF-β type I receptor immunostaining was restricted to the basal epidermal cells of normal skin with very little staining evident in the suprabasal layers (**Fig 3*A***). Minimal staining was observed in the dermis of normal skins. In contrast, intense type I receptor expression was seen throughout the thickened epidermal margin of the ulcers, including the suprabasal layers, and within fibroblasts in the dermal ulcer margin (**Fig 3*B***). Furthermore, strong immunoreactivity was observed in the ulcer bed with fibroblasts and inflammatory cells staining positive for TGF-β type I receptor (**Fig 3*C***). In the healing ulcers, positive staining for this receptor was again observed in the fibroblasts and inflammatory cells of the ulcer bed (**Fig 3*D***). The pattern of the type II receptor expression was markedly different to that observed for the type I receptor. Type II TGF-β receptor immunostaining was observed throughout the epidermis and in fibroblasts within the dermis of unwounded skins (**Fig 3*E***), a pattern similar to TGF-β1 and TGF-β3. Diminished staining was observed in the epidermal margins of the ulcers, although inflammatory cells and fibroblasts in the dermal margins were type II receptor positive (**Fig 3*F***). In marked contrast to the type I receptor, no type II receptor immunostaining was observed within the ulcers themselves (**Fig 3*G***). Immunofluorescent staining for TGF-β type II receptor in the healing ulcers revealed that fibroblasts within the ulcer were now staining positive for the receptor, a result similar to that observed for the ligands (**Fig 3*H***).

This absence of the TGF-βs and their receptor expression in nonhealing ulcers and the expression of these factors in healing ulcers were found to be consistent between patients. **Figure 4** shows staining patterns observed for TGF-β1 and the type II receptor. In all four nonhealing ulcers, no expression of TGF-β1 was observed (**Figs 1*C***, **4*A***, ***E***, ***I***), whereas, in marked contrast, staining was observed in healing ulcers from the same patients in fibroblasts and inflammatory cells (**Figs 1*D***, ***B***, ***F***, ***J***). Similarly, in all four nonhealing ulcers, no expression of the type II receptor was observed in the nonhealing ulcers (**Figs 2*G***, ***C***, ***G***, ***K***) whereas fibroblasts and inflammatory cells stained positive in the healing ulcers from the same patients (**Figs 2*H***, ***D***, ***H***, ***L***).

Quantitative competitive RT-PCR analysis The apparent loss of immunoreactivity for TGF-β1 and the type II receptor within chronic venous ulcers led us to further investigate the mRNA expression of these proteins using quantitative competitive RT-PCR (Tarnuzzer *et al*, 1996). Quantitative RT-PCR amplification was undertaken in the presence of increasing dilutions of a competitive template containing 337 bp sequences corresponding to TGF-β1, TGF-βRII, and β-actin mRNA (Tarnuzzer *et al*, 1996). The same reverse transcription reaction for each RNA sample was used to amplify all three genes under investigation. Amplification of the template produces a product of 337 bp that increases in intensity at the expense of the mRNA message in the biopsy samples with decreasing dilution. Expression of β-actin mRNA was detected in all normal and ulcer samples, confirming the integrity of the mRNA from the ulcer biopsies

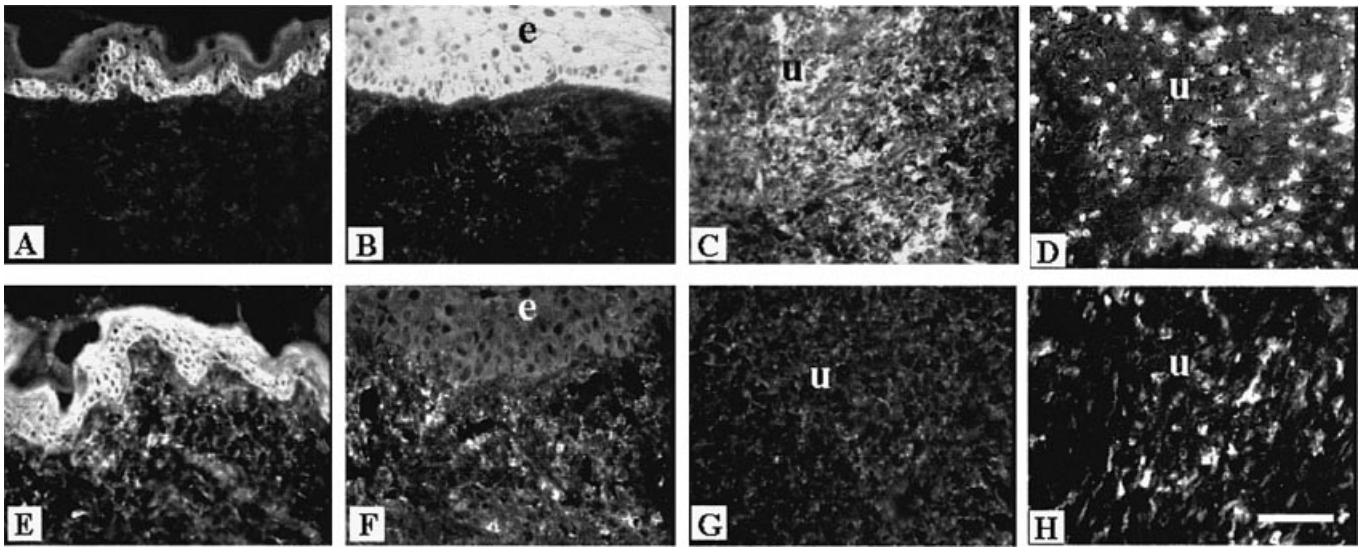

Figure 3. Immunofluorescent staining of the TGF-β type I and II receptors in unwounded normal skin and nonhealing and healing chronic venous ulcers. The unwounded normal skins, ulcer biopsies, and healing ulcer biopsies were stained using antibodies to TGF-βRI and TGF-βRII. Representative staining of the normal skin is shown in (*A*) and (*E*), ulcer edge (*B*, *F*), ulcer tissue (*C*, *G*) and healing ulcer (*D*, *H*). TGF-βRI immunofluorescent staining is shown in (*A*), (*B*), (*C*), and (*D*), and TGF-βRII in (*E*), (*F*), (*G*), and (*H*). *Scale bar*: 50 μm.

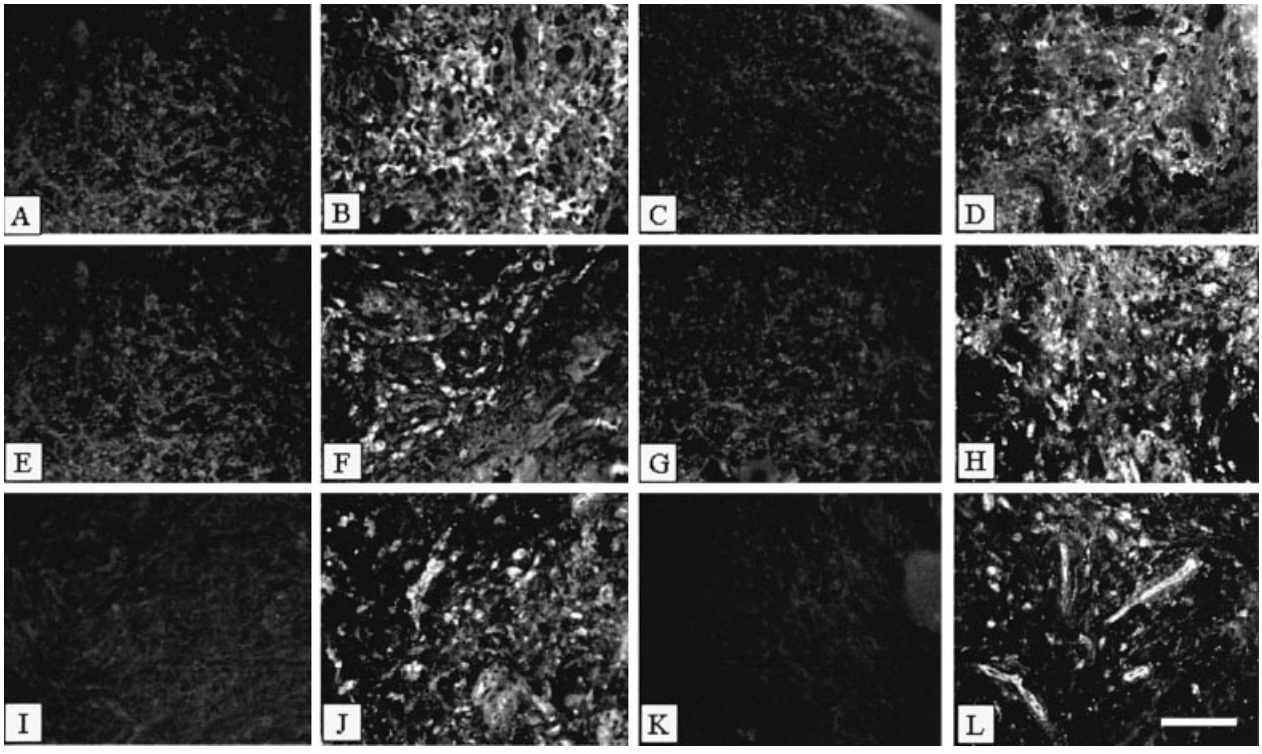

Figure 4. Immunofluorescent staining of TGF-β1, TGF-βRII in nonhealing and healing chronic venous ulcers. Nonhealing and healing venous ulcer biopsies taken from the same patients were stained using antibodies to TGF-βI and TGF-βRII. Three patients' biopsies are shown: Patient 1 (*A–D*), Patient 2 (*E–H*), Patient 3 (*I–L*). TGF-β1 staining is shown in nonhealing ulcers (*A*, *E*, *I*) and healing ulcers (*B*, *F*, *J*). TGF-βRII staining is shown in nonhealing ulcers (*C*, *G*, *K*) and healing ulcers (*D*, *H*, *L*). *Scale bar*: 50 μm.

(**Fig 5**). The number of copies of β-actin mRNA per μg total RNA ranged from 1.5×10^6 to 1.3×10^7 ($6.6 \times 10^6 \pm 3.9 \times 10^6$ mean ± SEM) in normal skin and from 1.0×10^6 to 2.6×10^7 ($63.9 \times 10^6 \pm 3.9 \times 10^6$ mean ± SEM) in ulcer tissue.

The expression of TGF-β1 mRNA was observed in all samples from normal skin and in five out of six of the biopsy samples from the base of the ulcers, producing the expected product at 442 bp (**Fig 5**). The mean copy number for the normal samples was $1.2 \times 10^5 \pm 1.0 \times 10^5$ (mean ± SEM) copies TGF-β1 mRNA per μg total RNA compared to $9.5 \times 10^4 \pm 5.7 \times 10^4$ (mean ± SEM) copies per μg total RNA extracted from the ulcer samples. PCR amplification of the TGF-βRII gene from RNA extracted

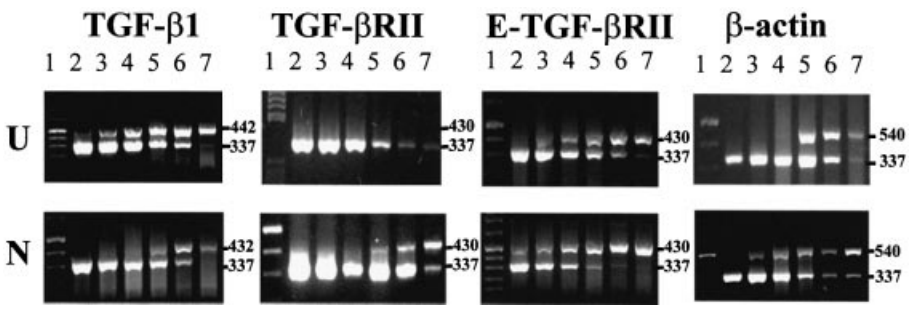

Figure 5. Ethidium bromide stained gels showing RT-PCR amplification of cellular RNA and template RNA for TGF-β1, TGF-β type II receptor and β-actin in normal skin and chronic venous leg ulcers. Representative gels showing gene products for TGF-β1 and TGF-βRII in nonhealing chronic ulcers (U) and normal skin samples (N) are shown. Enrichment of the reverse transcription reaction for TGF-βRII is shown in the third panel (E-TGF-βRII) for both the ulcers and normal skin samples. The markers are shown in *lane 1*, and *lanes 2–7* show amplification of decreasing amounts of reverse transcribed template RNA (2×10^9–2×10^4 copies, respectively) in the presence of 1 μg total RNA. The 337 bp band is the RT-PCR amplification of template RNA; the higher molecular weight band represents the amplification of TGF-β1 (442 bp), TGFβRII (430 bp), and β actin (540 bp).

from the ulcer biopsies revealed no expression in six of six wounds (**Fig 5**). Only the 337 bp template could be seen serving as a positive control for the PCR reactions. TGF-βRII PCR product at the expected 430 bp was observed in normal skin (**Fig 5**). To increase the sensitivity of the quantitative RT-PCR technique to detect very low levels of mRNA production we enriched the reverse transcription procedure by the use of a targeted 3′ primer for the gene of interest in the reverse transcription reaction. Using this selective procedure we found that all of the six ulcer tissues expressed low levels of the TGF-β type II receptor (**Fig 5**).

DISCUSSION

This study has revealed that the levels of all the TGF-β ligands are reduced in nonhealing venous leg ulcers and also that there is differential expression of the TGF-β receptors within these chronic wounds compared with normal, unwounded tissue. We observed no TGF-βRII receptor protein in any of the 12 chronic venous ulcers studied and low level expression of the TGF-βRII gene. Conversely, we observed expression of the type I receptor particularly at the wound margins and within the ulcers. Although the type I receptor staining was restricted to the basal cells within the epidermis of the normal unwounded skin, it was observed throughout the thickened epidermis in the skin surrounding the ulcers. The low level expression of the ligands and the absence of TGF-βRII protein in the ulcers could be important because for the TGF-β ligands to have any of their numerous wound healing activities they require the formation of a heterodimeric signaling complex (Derynck and Feng, 1997; Miyazono, 2000). The mechanism involved in TGF-β signaling involves the binding of the ligand to the type II receptor, which then recruits the type I receptor into the complex thereby forming a heteromeric complex of two TGF-βRIIs and two TGF-βRIs (Piek *et al*, 1999). Therefore, if one member of the TGF-β receptor complex is absent, as the TGF-βRII is in these nonhealing venous ulcers, we would expect receptor signaling to be compromised and neither endogenous nor exogenous TGF-β ligand would have its expected effects on wound repair. In support of our *in vivo* observations, *in vitro* studies have shown that venous-ulcer-derived fibroblasts are unresponsive to TGF-β1 and that this responsiveness is associated with a 4-fold decrease in TGF-β type II receptors (Hasan *et al*, 1997).

The appearance of the epidermis surrounding the ulcers was very different to that seen in normal skin or in acute wounds. Markedly thickened, acanthotic, hyperplastic epidermis was observed at the ulcer wound margins. This type of epidermal appearance has been interpreted to be regenerative or proliferative rather than migratory (Falanga *et al*, 1994). As TGF-β switches keratinocytes from a differentiating to a regenerative phenotype (Nickoloff *et al*, 1988), reduced TGF-β activity in the epidermal wound margins may result in continued proliferation and differentiation, rather than migration across the wound bed. It has been revealed that the loss of TGF-β1, or SMAD3, a component of the TGF-β signaling system, actually enhances reepithelialization in acute wounds (Koch *et al* 2000; Ashcroft *et al*, 1999; respectively). Therefore the reduced epithelialization observed in chronic venous leg ulcers is unlikely to be due only to the reduced expression of the TGF-βs or their receptors. In fact it is likely that other factors may contribute significantly to the inhibition of wound closure in ulcers compared to acute wounds. For example, TGF-β stimulates fibronectin production in fibroblasts (Ignotz and Massagué, 1986) and keratinocytes (Clark *et al*, 1982), and studies have revealed that fibronectin is absent in the matrix of chronic nonhealing ulcers (Herrick *et al*, 1992). As fibronectin is required within the provisional matrix for epithelial migration to occur (Clark *et al*, 1982), the reduced expression of the TGF-βs in conjunction with low expression of fibronectin could inhibit the reepithelialization of chronic wounds and could lead to the thickened epidermis observed surrounding the ulcers. Interestingly, although TGF-β1 and TGF-β3 staining was observed in epidermal cells adjacent to the ulcers, TGF-β2 staining was restricted to the suprabasal epidermal cells and was much reduced in the surrounding skin of the ulcers compared to normal unwounded skin. The reason for this difference in ligand expression may reflect different functions for the TGF-βs in epidermal cells and may further point to the different wound healing activities that the TGF-β ligands possess (Shah *et al*, 1995).

The method of quantitative competitive RT-PCR, developed by Tarnuzzer *et al* (1996), was used to detect the expression of mRNA for TGF-β1, TGF-βRII, and β-actin. This procedure has been successfully used to determine mRNA levels in dermal wound biopsies (Tarnuzzer and Schultz, 1996; Ashcroft *et al*, 1997) and requires only low levels of mRNA, which was an important consideration due to the size of the biopsies taken from the venous ulcers. Although large variations were observed between mRNA obtained from different ulcer patients, due perhaps to variations in ulcer age and size, we observed expression of mRNA for TGF-β1 in all of the ulcer samples. As negligible protein was detected using immunofluorescent staining, however, the mRNA may not be being transcribed and translated into protein or the peptide may be degraded by proteases within the chronic wound environment. Previous studies have shown that there are high levels of proteases in chronic wound fluid (Yager *et al*, 1996; Herrick *et al*, 1997), and additional studies have also shown that chronic wound fluid can readily degrade growth factors and their receptors (Bennett and Schultz, 1993).

In contrast to the readily detectable expression of mRNA for TGF-β1, no expression of the TGF-βRII was seen in any of the six

ulcers investigated using conventional RT-PCR, but when an enrichment protocol to select for TGF-βRII was used gene expression was observed. This indicates that either the cells within the ulcers have reduced ability to produce mRNA for the type II receptor or that the mRNA is unstable and being degraded. Additionally, the absence of protein expression for the type II receptor in nonhealing ulcers could indicate that the mechanism involved may also be one of transcriptional suppression, particularly as re-expression of this receptor is observed in healing ulcers. Diminished cellular responsiveness to TGF-βs has frequently been correlated with decreased transcription of the type II receptor gene (Bae *et al*, 1995). In keratinocytes, the mechanism involved in this transcriptional downregulation of the TGF-βRII gene has been shown to involve decreased interaction between transcription factor proteins and their target sequences within positive regulatory elements of the TGF-βRII promoter (Kim *et al*, 1997). Further characterization of the promoter region of the TGF-βRII gene has also revealed the existence of a negative *cis*-regulatory element that can inhibit transcription of this gene (Song *et al*, 2001).

Reduced expression of the TGF-β type II receptor has been observed in other wound repair pathologies. Mice treated with glucocorticoids have impaired wound healing responses and studies have revealed that there is differential expression of the TGF-β receptors in these wounds with low levels of the type II receptor detected (Frank *et al*, 1996). Also in fibrotic lesions associated with atherosclerosis, the normal antiproliferative effects of TGF-β1 on vascular cells is reduced and there is a concomitant reduction in the expression of the type II receptor (McCaffrey, 2000).

The addition of exogenous TGF-βs to chronic wounds has been suggested as a potential therapeutic modality. This is due to their ability to enhance the normal repair process in animal models by increasing the degree of cellularity and the rate of angiogenesis, stimulating keratinocyte migration and increasing the amount of collagen accumulated. Despite several clinical studies having been performed using recombinant TGF-βs, however, to date no TGF-β based preparation is on the market or near market approval. Suggestions for this lack of significant clinical efficacy have included problems with the delivery vehicle, the dose, or the length of application (Robson, 1997). Given the results obtained in this study we propose that another factor to consider is the level of TGF-β receptor expression in chronic dermal wounds. Importantly, following the implementation of good wound management the profile of the TGF-βs and their receptors more closely resembles that observed in the acute wound (Frank *et al*, 1996; Gold *et al*, 1997).

In summary, this study has revealed that the levels of all the TGF-β ligands are reduced in venous leg ulcers and also that there is differential expression of the TGF-β receptors within these chronic wounds compared with normal, unwounded tissue. We postulate that the absence of a viable receptor complex could be important in the chronicity of venous ulceration and may account for the reduced proliferation and increased degradation that is symptomatic of chronic wound healing. Once an ulcer has entered a healing phase expression of TGF-β and its receptors is upregulated and at this stage exogenous addition of growth factors may be more effective. This could be of particular importance in the clinical trialing of growth factors for their effects on impaired wound healing.

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